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β-Adrenergic regulation of uncoupling protein expression in swine

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Abstract

This study examined the β-adrenergic regulation of uncoupling protein (UCP) 2 and UCP3 gene expression in porcine tissues. *In vitro* experiments examined changes in UCP2 and UCP3 gene expression in middle (MSQ) and outer (OSQ) subcutaneous adipose tissues from crossbred neutered male pigs. Incubation of tissue slices (24 h) with 0 to 1000 nM isoproterenol increased UCP2 and UCP3 mRNA abundance in MSQ and OSQ, relative to 18S rRNA ($P<0.05$). For the *in vivo* experiment, nine randomly selected pigs (80 kg) were presented with a diet supplemented with 10.0 ppm ractopamine for 2 weeks. Another eight pigs were maintained on a control diet. Dietary ractopamine did not affect adipose UCP2 or UCP3 gene expression ($P>0.05$). However, UCP2 mRNA abundance was depressed in semitendinosus white (STW, $P<0.05$) and semitendinosus red (STR, $P<0.001$) by ractopamine feeding. Also, ractopamine decreased UCP3 mRNA abundance by 28% in STW ($P<0.05$). The *in vitro* data suggest that β-adrenergic agonists directly affect adipose tissue UCP expression, although these adipose effects can be masked by the *in vivo* physiology. The *in vivo* data indicate that β-adrenergic agonists may function in regulating UCP2 and UCP3 expression in selected muscles.

Keywords: Adipose tissue; β-adrenergic agonist; Muscle swine; Uncoupling protein

1. Introduction

Uncoupling protein 1 (UCP1) is a molecule with the capacity to uncouple mitochondrial respiration by dissipating the transmembrane electrochemical potential through transporting protons from the intermembrane space back toward the matrix of the mitochondria, promoting a proton leakage and generating energy (Ricquier and Bouillaud, 2000). UCP2 and UCP3 share 55% and 57% amino acid identity with UCP1 and 73% with each other (Boss et al., 1997; Fleury et al., 1997). Uncoupling protein 2 has been associated with metabolism of free radicals and mild uncoupling activity (Jezek and Garlid, 1998). Using UCP3 knockout mice, UCP3 has been associated with changes in mitochondrial energy production that suggests uncoupling activity specific to skeletal muscle (Cline et al., 2001); while other studies have indicated a more specific role for UCP3 with regulation of fatty acid metabolism and ATP-dependent processes (Jezek and Garlid, 1998; Erlanson-Albertsson, 2003). Damon et al. (2000) and Spurlock et al. (2001) have reported that both UCP2 and UCP3 are expressed in porcine skeletal muscle and adipose tissue.

The sympathetic nervous system has been demonstrated to be an important regulator of adaptive thermogenesis and UCP expression (Sivitz et al., 1999; Lowell and Bachman, 2003), although many other hormones can also impact UCP expression (Damon et al., 2000; Ricquier et al., 2000; Ramsay and Rosebrough, 2005). Use of β-adrenergic agonists *in vitro* has demonstrated that UCP2 expression can be elevated in human adipocytes (Shi et al., 2002) or 3T3-L1 adipocytes (Yoshitomi et al., 1999), despite differences in β-adrenergic receptor subtypes. However, no *in vitro* studies have examined the potential for β-adrenergic regulation of UCP3 in adipose tissue. While β-adrenergic regulation of porcine adipose tissue metabolism has been clearly defined (Mersmann, 1998), the putative role for β-adrenergic regulation of uncoupling proteins in porcine adipose tissue has not been identified.

Secondly, the *in vivo* role of β-adrenergic agonists in the regulation of UCP2 and UCP3 is unclear. This has been

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complicated by species differences in the population of β adrenergic receptor subtypes within tissues (McNeel and Mersmann, 1999). For example, the β3 adrenergic receptor subtype is the overwhelming contributor to adrenergic regulation in the rat adipocyte (Lafontan, 1994) while the pig (McNeel and Mersmann, 1999) and human (Lafontan and Berlan, 1993) have minimal β3 receptor subpopulations. Sivitz et al. (1999) found no effect of pharmacological systemic inhibition of catecholamine synthesis on rat epididymal adipose UCP2 expression while elevating UCP3 expression. In contrast, Emilsson et al. (1998) reported the β3-adrenergic agonist BRL 35135 induced rat epididymal adipose UCP2 and UCP3, as also reported for the β3-adrenergic agonist CL316243 (1997). Yoshitomi et al. (1998) demonstrated that the β3-adrenergic agonist CL316243 increased mouse epididymal adipose tissue UCP3 expression but was ineffective on UCP2 expression. Whether this variability in UCP2 and UCP3 expression to β-adrenergic agonists is due to the particular agonist, species or experimental design cannot be ascertained.

The recent approval and agricultural use of the β-adrenergic agonist ractopamine hydrochloride (Elanco Animal Health, Greenfield, IN) provides a useful tool to examine the in vivo β-adrenergic regulation of UCP2 and UCP3 within the adipose tissue of swine. Ractopamine induces the partitioning of energy away from adipose tissue and toward muscle protein synthesis and lean deposition (Mersmann, 1998; Moody et al., 2000). At the cellular level, ractopamine has been demonstrated to inhibit lipogenesis *in vitro* and stimulate lipolysis in porcine adipocytes following binding to β_1 and β_2 adrenergic receptors (Mills and Liu, 1990; Spurlock et al., 1993; Mills et al., 2003). Thus, the present study was designed to determine if the expression of the porcine uncoupling proteins is regulated *in vivo* and *in vitro* by β-adrenergic agonists.

2. Materials and methods

2.1. In vitro expression

For in vitro experiments, subcutaneous adipose tissue was collected from four crossbred barrows (*Sus scrofa*, Yorkshire x Landrace) at approximately 110 kg bwt. Dorsal subcutaneous adipose tissue samples from between the second and fourth thoracic vertebrae were acquired following euthanasia by electrical stunning and exsanguination according to procedures approved by the Institutional Area Animal Use and Care Committee. Adipose tissue was prepared for chronic tissue slice incubation according to methods previously described (Ramsay and Rosebrough, 2005). Briefly, dissected middle adipose tissue (MSQ) and outer subcutaneous adipose tissue (OSQ) were diced into 1×4 cm strips and placed in Hanks buffer (37 °C, pH 7.4). Adipose tissue strips were then dissected clean of any extraneous muscle tissue and further separated into 1 cm cubes in a laminar flow hood. Adipose tissue explants (approximately 100 mg) were prepared by slicing tissue cubes with a Stadie–Riggs microtome. Tissue slices (400 μm thickness) were rinsed twice with fresh Hanks buffer (37 °C, pH 7.4), blotted free of excess liquid and weighed. Tissue slices were then transferred to 12 well tissue culture plates containing 1 mL of DMEM/F12 with 25 mM HEPES, 0.5% BSA, pH 7.4 and the various hormone supplements of interest. Triplicate tissue slices for each pig were incubated with each medium (basal medium, isoproterenol supplemented medium) in a tissue culture incubator at 37 °C with 95% air/5% CO_2 for 24 h. At the end of incubation period, tissue samples from these incubations were blotted dry and frozen in liquid nitrogen for later analysis of gene expression by real-time PCR analysis.

Previous research (Ramsay and Rosebrough, 2005) has demonstrated that metabolic viability of these tissue incubations is maintained for at least 24 h. Stability of UCP2 mRNA abundance under basal conditions is maintained when comparing tissue incubated for 24 h relative to 2 h (94±8% of 2 h incubation; $P>0.05$, $n=5$). Stability of UCP3 mRNA is slightly less with mRNA abundance decreasing by approximately 8% but still not different from 2 h incubations (92±12%; $P>0.05$, $n=5$).

Isoproterenol was used for *in vitro* experiments as it is a universal β-receptor agonist in swine (Mersmann, 1998). Purified ractopamine was not available for these *in vitro* experiments, but functions through β1 and β2 adrenergic receptors (Mills et al., 2003). Isoproterenol was prepared fresh daily for the experimental trials. Isoproterenol was solubilized in distilled water (10 μg/mL) and filtered. Serial dilutions of 10, 100 and 1000 ng/mL were supplemented to medium for 24 h incubations and compared to a basal medium (control).

2.2. Real-time PCR analysis of gene expression

The expression of UCP2 and UCP3 was assessed in tissue slices following 24 h of incubation in serial dilutions of isoproterenol by real time PCR analysis. Tissue samples from these incubations were blotted and transferred to microfuge tubes with subsequent freezing in liquid nitrogen and storage at −80 °C prior to analysis for uncoupling protein gene expression.

Total RNA was isolated using TRI Reagent according to the manufacturer's protocol (Sigma-Aldrich, St. Louis, MO, USA). RNA integrity was assessed via agarose gel electrophoresis and RNA concentration and purity were determined spectrophotometrically using A260 and A280 measurements.

Reverse transcription (RT) and real time PCR analysis were performed in a single tube using the QuantiTect SYBR Green RT-PCR protocol (Qiagen, Inc., Valencia, CA, USA) according to procedures previously published by this laboratory (Ramsay and Rosebrough, 2005). Reactions (25 μL) consisted of 1 μg total RNA, 12.5 μL QuantiTect SYBR Green RT-PCR Master Mix, 0.5 μM primers, 0.25 μL QuantiTect RT Mix, 9 μL RNase-free H_2O. Thermal cycling and data acquisition were performed with a DNA Engine Opticon Continuous Fluorescence Detection System (MJ Research, Waltham, MA, USA). Thermal cycling parameters were as follows: 1 cycle 50 °C for 30 min (reverse transcription), 1 cycle 95 °C for 15 min (PCR activation), followed by 30 cycles, 94 °C for 15 s, 58 °C for 30 s, 72 °C for 30 s, with a final extension at 72 °C for 8 min. Melting curve analysis was performed on all real-time PCR reactions to confirm specificity and identity of the real time PCR products. Specificity of real time PCR products was further confirmed by agarose gel electrophoresis. The one-step real time PCR for UCP2, UCP3 and 18S were optimized for linearity (exponential amplification) from >20 to <30 cycles under the conditions described above.

The following primers were used for generating 210-base-pair fragments corresponding to a part of the translated UCP2 sequence: 5′-CTGCAGATCCAGGGAGAAAG-3′ (forward), 5′-GCTTGACGGAGTCGTAGAGG-3′ (reverse). The primers for UCP3 were used to generate 200 base-pair fragments corresponding to a part of the translated sequence: 5′-ACGATG-GATGCCTACAGGAC-3′ (forward), 5′-TCCGAAGGCAGA-GACAAAGT-3′ (reverse). The primers for 18S ribosomal RNA were purchased (QuantumRNA™ Universal 18S Internal Standard; Ambion, Inc; Austin, TX, USA). The UCP2 and UCP3 amplicons were excised from an agarose gel, re-amplified, and run through a GenElute PCR clean-up kit (Sigma-Aldrich). UCP2 and UCP3 amplicons were sequenced to confirm identity using automated fluorescent DNA sequencing (ABI 310, Perkin Elmer Applied Biosystems, Foster City, CA, USA).

2.3. Quantification of gene expression

At the end of the PCR, baseline and threshold crossing values (C_T) for UCP2, UCP3 and 18S were calculated using the Opticon Monitor Software (Version 1.06; MJ Research, Waltham, MA) and the C_T values were exported to Microsoft Excel for analysis. The relative expression of UCP2 and UCP3 mRNA were calculated using the comparative C_T method according to manufacturer's literature (MJ Research). The C_T values were transformed to their respective antilogarithmic values. The relative amount of UCP2 or UCP3 mRNA, standardized against the amount of 18S rRNA, in adipose tissue explants was expressed as $\Delta C_T = [C_T\text{UCP} - C_T\text{18S}]$. The ratio of UCP mRNA/18S rRNA, i.e. the relative UCP expression, was then calculated as 2^{-CT}. Data are presented as the % change in amplicon number relative to the control group amplicon number for each gene. Values are presented as the mean ± SEM of determinations from 4 individual animals.

2.4. In vivo expression

For *in vivo* experiments, seventeen crossbred barrows (Yorkshire x Landrace) were individually penned in environmentally controlled housing at 55 kg. Animals were individually fed a basal diet containing 18% CP, 1.2% lysine, and 3.5 Mcal of DE/kg *ad libitum*. This basal diet has been previously described (Ramsay and Richards, 2005).

At 80 kg, nine randomly selected pigs were switched to the basal diet supplemented with 10.0 ppm ractopamine (Paylean, Elanco Animal Health, Greenfield, IN). This dosage was based upon previous studies in pigs of this size (Armstrong et al., 2004; See et al., 2004). The other eight pigs served as controls and were maintained on the basal diet (Control). Animals were fed *ad libitum*. Animals were maintained on treatment for 2 weeks. A blood sample was obtained from each pig in the fed state on day 14 of treatment at 1500 for hormone and metabolite analysis. Animals were euthanized on day 15 at 0800.

Various tissues were acquired following euthanasia by electrical stunning and exsanguination according to procedures approved by the Institutional Area Animal Use and Care Committee. Dorsal subcutaneous adipose tissue samples were collected between the second and fourth thoracic vertebrae and subsequently OSQ and MSQ were separated, diced and frozen in liquid nitrogen. In addition samples of liver, leaf (perirenal) fat, longissimus (LM) and semitendinosus (ST) were collected, diced and frozen in liquid nitrogen. Outer and middle subcutaneous adipose tissues were separated for analysis because of their known differences in metabolic activity, while leaf fat has a different metabolic profile from either subcutaneous adipose tissue (Anderson et al., 1972; Rule et al., 1989; Budd et al., 1994). The LM and ST were selected as they have been demonstrated to respond to ractopamine (Adeola et al., 1992a,b; Spurlock et al., 1993; Depreux et al., 2002). Outer white (STW) and inner red portions of the ST (STR) were separated to permit analysis of potential differences in UCP expression with fiber type (Spurlock et al., 2001).

The hot carcass weight was recorded following removal of the head and viscera. The carcass was separated into right and left sides. The right side of the carcass was chilled at 5 °C for 2 to 3 days and weighed. Carcass length was measured from the first rib to the aitch bone. Backfat thickness was measured at the first rib, last rib, P2 (thickness at the fabrication of the last rib) and the last lumbar vertebra. At the 10th–11th rib interface, LM muscle area was measured by tracing of the perimeter of the exposed cross-section of the muscle and then a retracing using a digitizing table; the area was computed based on the Sigma Scan (Aspire Software Int., Leesburg, VA, USA) procedure.

2.5. Hormone and metabolite analysis

Serum was prepared from blood collected on d14 of treatment as previously described. Concentrations of T3, T4, cortisol were determined by homologous RIA using commercial kits (Diagnostics Products Co., Los Angeles, CA, USA). Intra-assay CV was 4.9% for T3, 4.6% for T4 and 3.4% for cortisol. Interassay CV was 6.6% for T3, 8.1% for T4 and 5.2% for cortisol. Serum insulin was measured using a homologus RIA kit using human standards (Linco Research Inc., St. Charles, MO, USA), previously utilized for analysis of pig insulin (Ramsay et al., 2004). Intraassay CV for insulin was 7.0%, while the interassay CV was 9.4%. Serum IGF-1 was assayed using a heterologous immunoradiometric kit (Diagnostics Systems Laboratory Inc., Webster, TX, USA) following acid-ethanol extraction and previously validated for swine (Balaji et al., 2000). Intraassay CV for IGF-1 was 6.1% while the interassay CV was 3.7%. Serum glucose (Thermo DMA, Louisville, CO, USA), triglycerides (DMA, Arlington, TX, USA) and non-esterified free fatty acids (WakoChemical Co., Richmond, VA, USA) were determined with kit based assays.

2.6. Gene expression analyses by reverse transcription-PCR

Total RNA was isolated using TRI Reagent according to the manufacturer's protocol (Sigma-Aldrich). Integrity of RNA was assessed via agarose gel electrophoresis and RNA concentration and purity were determined spectrophotometrically using A260 and A280 measurements. Reverse transcription (RT) reactions (20 μL) consisted of 1 μg total RNA, 50 U SuperScript II reverse transcriptase (Invitrogen/Life Technologies, Carlsbad, CA, USA), 40 U of an RNAse inhibitor (Invitrogen/Life Technologies), 0.5 mmol/L dNTP, and 100 ng random hexamer primers. Polymerase chain reaction was performed in 25 μL containing 20 mmol/L Tris–HCl, pH 8.4, 50 mmol/L KCl, 1.0 μL of the RT reaction, 1.0 U of Platinum Taq DNA polymerase (Hot Start, Invitrogen/Life Technologies), 0.2 mmol/L dNTP, 2.0 mmol/L Mg^{+} (Invitrogen/Life Technologies), 10 pmol each of the UCP2 and UCP3 specific primers and 10 pmol of an appropriate mixture of primers and competimers specific for 18S rRNA (QuantumRNA™ Universal 18S Internal Standard; Ambion, Inc; Austin, TX, USA). Thermal cycling protocol was as follows: 1 cycle 94 °C for 2 min, followed by 35 cycles, 94 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min with a final extension at 72 °C for 8 min.

The following primers were used for generating 210-base-pair PCR products corresponding to a portion of the pig UCP2 coding sequence: 5′-CTGCAGATCCAGGGAGAAAG-3′ (forward), 5′-GCTTGACGGAGTCGTAGAGG-3′ (reverse). The primers for UCP3 were used to generate a 200 base-pair product: 5′-ACGATGGATGCCTACAGGAC-3′ (forward), 5′-TCCGAAGGCAGAGACAAAGT-3′ (reverse). The UCP2 and UCP3 amplicons were excised from an agarose gel, re-amplified, and run through a GenElute PCR clean-up kit (Sigma-Aldrich). The amplicons were subsequently sequenced to confirm identity using automated fluorescent DNA sequencing (ABI 310, Perkin Elmer Applied Biosystems, Foster City, CA, USA).

2.7. Capillary electrophoresis with laser-induced fluorescence detection

Aliquots (2 μL) of RT-PCR samples were diluted 1:100 with deionized water before capillary electrophoresis with laser-induced fluorescence detection (CE/LIF). A detailed description and validation of the CE/LIF technique used in this study for quantitative analysis of gene expression was reported previously (Richards and Poch, 2002). Briefly, a P/ACE MDQ CE instrument (Beckman Coulter, Fullerton, CA, USA) equipped with an argon ion LIF detector was used. Capillaries were 75 μm i.d. ×32 cm μSIL-DNA (Agilent Technologies, Folsom, CA). EnhanCE dye (Beckman Coulter) was added to the DNA separation buffer (Sigma-Aldrich) to a final concentration of 0.5 μg/mL. Samples were loaded by electrokinetic injection at 3.5 kV for 3.5 s and run in reverse polarity at 8.1 kV for 5 min. Integrated peak area for the PCR products separated by CE was calculated using P/ACE MDQ software (Beckman Coulter).

2.8. Quantification of mRNA abundance

The relative level of mRNA abundance was determined as the ratio of integrated peak area for each individual gene PCR product relative to that of a coamplified 18S internal standard (QuantumRNA™ Universal 18S Internal Standard; Ambion, Inc). Values are presented as the mean±SEM of eight to nine individual determinations.

2.9. Statistical analysis

Data were analyzed by analysis of variance using SigmaStat software (SPSS Science, Chicago, IL, USA). Mean separation was analyzed using Student–Newman–Keuls test. Means were defined as significantly different at $P<0.05$.

3. Results

Twenty four hour incubation of subcutaneous adipose tissue slices with isoproterenol was sufficient time to produce changes

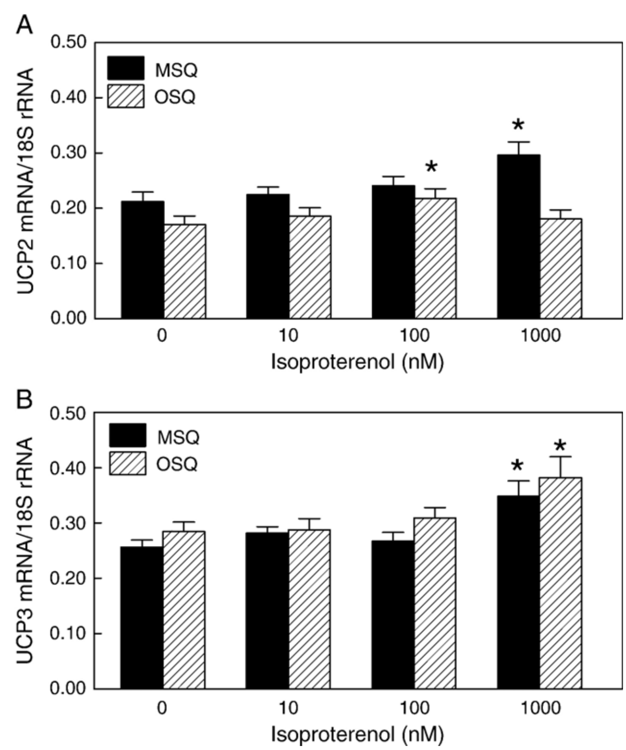

Fig. 1. A. Relative UCP2 mRNA abundance in subcutaneous adipose tissues from swine incubated in the presence of isoproterenol (0 to 1000 nM) for 24 h followed by extraction for total RNA and subsequent RT-PCR analysis for UCP2 mRNA abundance. Data are expressed as the mean ratio±SEM of specific UCP2 mRNA:18S rRNA for tissue from 4 pigs in each group. *Isoproterenol effect ($P<0.05$, $n=4$). MSQ = middle subcutaneous adipose tissue; OSQ = outer subcutaneous adipose tissue. B. Relative UCP3 mRNA abundance in subcutaneous adipose tissues from swine incubated in the presence of isoproterenol (0 to 1000 nM) for 24 h followed by extraction for total RNA and subsequent RT-PCR analysis for UCP3 mRNA abundance. Data are expressed as the mean ratio±SEM of specific UCP3 mRNA:18S rRNA for tissue from 4 pigs in each group. *Isorproterenol effect ($P<0.05$, $n=4$). MSQ=middle subcutaneous adipose tissue; OSQ=outer subcutaneous adipose tissue.

Table 1
Weight gain and carcass parameters for barrows fed a basal diet (control) or supplemented ractopamine (10 ppm) during a 2-week trial

Carcass parameter	Control	Ractopamine
Live Wt Gain (kg)	11.6±1.4	16.1±1.0*
Carcass Wt (kg)		
Whole	75.5±1.0	79.4±1.1*
Half	35.0±0.8	39.5±1.1*
Carcass right side		
Chilled Wt (kg)	38.4±0.6	40.7±0.7
Length (cm)	760.0±11.1	766.8±7.3
Fat Thickness (mm)		
1st Rib	36.3±3.0	37.6±4.3
Last Rib	26.4±1.4	23.6±2.6
Last Lumbar	23.1±3.0	22.0±2.7
P2	28.3±2.1	22.3±1.9*
Longissimus area (cm2)	33.7±1.6	36.1±1.0

* $P<0.05$, $n=8$ to 9 animals.

in subcutaneous adipose tissue gene expression. Both OSQ and MSQ showed increases in UCP2 expression with isoproterenol treatment (Fig. 1A), although the maximal response to isoproterenol was attained at 100 nM in OSQ ($P<0.05$) while it was not reached by 1000 nM in the MSQ ($P<0.01$). The abundance of UCP2 mRNA was similar in OSQ and MSQ in this *in vitro* study ($P>0.05$). Isoproterenol (1000 nM) induced a 36% increase in MSQ UCP3 mRNA abundance relative to 0 nM isoproterenol incubations ($P<0.01$, Fig. 1B). Uncoupling protein 3 expression was induced to a similar extent, 34% ($P<0.01$), in OSQ by 1000 nM isoproterenol relative to control incubations (0 nM isoproterenol). Lower concentrations of isoproterenol were ineffective in altering UCP3 expression in subcutaneous adipose tissue slices relative to control incubations ($P>0.05$). Relative UCP3 expression did not differ between MSQ and OSQ ($P>0.05$). Overall levels of UCP3 mRNA abundance were higher than UCP2 mRNA abundance within both subcutaneous regions of adipose tissue ($P<0.05$).

Supplementing ractopamine to the feed for 2 weeks produced a 39% greater weight gain in treated swine than in control animals ($P<0.05$; Table 1). Carcass analysis demonstrated that this body weight gain was reflected in an increase in hot carcass weight of ractopamine treated pigs relative to animals fed the basal diet ($P<0.05$, Table 1). Carcass length and chilled weight were unaffected by 2 weeks of ractopamine treatment ($P>0.05$). Only the P2 measurement of subcutaneous adipose thickness was affected by ractopamine treatment among the measurements of backfat thickness. The P2 thickness was reduced by 22% with dietary ractopamine treatment ($P<0.05$).

Analysis of serum from these swine could not demonstrate an effect of dietary ractopamine on serum IGF-I (control: 376±30 ng/mL, ractopamine: 425±35 ng/mL; $P>0.05$), insulin (control: 14.7±3.1 µU/mL, ractopamine: 13.8±2.7 µU/mL; $P>0.05$), T4 (control: 45±4 µg/L, ractopamine: 39±3 µg/L; $P>0.05$), or T3 (control: 456±76 ng/L, ractopamine: 464±34 ng/L; $P>0.05$). Serum cortisol was not significantly increased by ractopamine treatment (control: 57±11 µg/L, ractopamine: 87±11 µg/L; $P=0.063$). Serum glucose concentration was not altered by ractopamine treatment (control: 5.09±0.28 mMol/L, ractopamine: 4.81±0.22 mMol/L; $P>0.05$). Neither serum triglycerides (control: 35±5 mg/L, ractopamine: 37±5 mg/L; $P>0.05$) nor NEFA (control: 48.0±4.8 µEq/L, ractopamine: 40.6±1.9 µEq/L; $P>0.05$) were affected by ractopamine treatment.

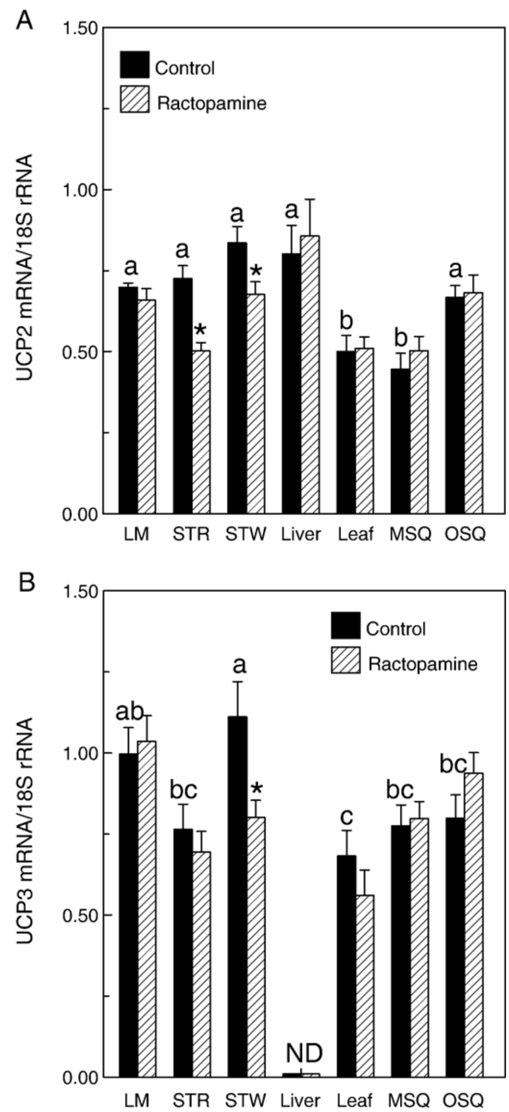

Fig. 2. A. Relative UCP2 mRNA abundance in tissues from swine following 14 days of dietary ractopamine (10 ppm) treatment followed by extraction for total RNA and subsequent RT-PCR analysis for UCP2 mRNA abundance. Data are expressed as the mean ratio±SEM of specific UCP2 mRNA:18S rRNA for 8–9 pigs in each group. * Ractopamine effect ($P<0.05$, $n=8$–9). [ab]Means not sharing a common letter are significantly different. ($P<0.05$, $n=8$ to 9 animals). LM=longissimus; STR=semitendinosus red portion; STW=semitendinosus white portion; LF=leaf fat; MSQ=middle subcutaneous adipose tissue; OSQ=outer subcutaneous adipose tissue. B. Relative UCP3 mRNA abundance in tissues from swine following 14 days of dietary ractopamine (10 ppm) treatment followed by extraction for total RNA and subsequent RT-PCR analysis for UCP3 mRNA abundance. Data are expressed as the mean ratio±SEM of specific UCP3 mRNA:18S rRNA for 8–9 pigs in each group. * Ractopamine effect ($P<0.05$, $n=8$–9 animals). [abc] Means not sharing a common letter are significantly different. ($P<0.05$, $n=8$–9). LM=longissimus; STR=semitendinosus red portion; STW=semitendinosus white portion; LF=leaf fat; MSQ=middle subcutaneous adipose tissue; OSQ=outer subcutaneous adipose tissue. ND=not detectable.

The UCP2 mRNA abundance was decreased by 20% in STW ($P<0.05$) and 31% in STR ($P<0.001$) of ractopamine treated swine relative to control animals fed the basal diet (Fig. 2A). In contrast, LM did not demonstrate a change in UCP2 mRNA abundance with ractopamine treatment ($P>0.05$). Abundance of UCP2 mRNA in all three sites of adipose tissue deposition was not affected by dietary ractopamine treatment ($P>0.05$ for leaf fat; $P>0.05$ for MSQ; $P>0.05$ for OSQ). Hepatic UCP2 expression was also not affected by ractopamine ($P>0.05$). Comparison of UCP2 mRNA abundance among tissues demonstrated that expression was least in MSQ and leaf fat ($P<0.001$). Uncoupling protein 2 mRNA abundance in all other tissues was similar to liver ($P>0.05$).

Uncoupling protein 3 mRNA abundance was 28% less in STW of ractopamine treated swine than control swine (Fig. 2B; $P<0.05$). Dietary ractopamine treatment did not alter UCP3 expression in any other tissue examined ($P>0.05$). Liver did not express detectable UCP3 mRNA. Comparison of UCP3 mRNA abundance among tissues showed that UCP3 mRNA levels were highest in LM and STW and similar amongst all other tissues transcribing UCP3 mRNA ($P<0.001$).

4. Discussion

Isoproterenol is a non-selective βAR agonist that can alter the expression of UCP2 and/or UCP3 in human adipocytes (Shi et al., 2002), mouse adipocyte (Yoshitomi et al., 1999), mouse brown adipocyte (Viengchareun et al., 2001) and rat skeletal muscle (Nagase et al., 2001) cell lines. The human adipocyte expresses all three subtypes of β-adrenergic receptor subtypes, but β1 and β2 adrenergic receptor activity predominate (Lafontan and Berlan, 1993), while the rodent tissues express primarily β3 adrenergic receptors. The present study is the first to demonstrate that isoproterenol can induce UCP2 and UCP3 in subcutaneous adipose tissues of swine *in vitro*, a tissue which contains primarily β1 adrenergic receptors with limited β3 adrenergic receptors (McNeel and Mersmann, 1999). The absence of any differences between the MSQ and OSQ in their response to isoproterenol was not unexpected as the majority of hormone responses by these two sites of adipose tissue deposition are similar (Ramsay and Rosebrough, 2005).

Only two previous *in vitro* studies have examined β-adrenergic regulation of UCP2 expression in adipocytes (Yoshitomi et al., 1999; Shi et al., 2002). Shi et al. (2002) demonstrated that 10 nM isoproterenol could increase UCP2 mRNA abundance by 80–130% in human adipocytes. The present study could not detect an effect of 10 nM isoproterenol, a minimum of 100 nM was required to detect a response with the present culture conditions. Both species express β1 and β2 adrenergic receptors at a much higher level than β3 adrenergic receptors (Lafontan and Berlan, 1993; McNeel and Mersmann, 1999). Whether this difference in sensitivity to isoproterenol between studies is due to a difference between culture conditions or a difference in binding affinities is unknown. The increase in UCP2 mRNA abundance with isoproterenol treatment in the present study was similar to the increase in UCP2 expression obtained with 2 μM isoproterenol treatment of the mouse adipocyte cell line, 3T3-L1 (Yoshitomi et al., 1999) which expresses β3 adrenergic receptors predominantly (Lefrere et al., 2002).

No previous *in vitro* study has examined the regulation of UCP3 expression by β-adrenergic agonists in white adipocytes. However, UCP3 mRNA abundance was reduced by 75% with 1 μM isoproterenol treatment in the T37i brown adipocyte cell line (Viengchareun et al., 2001). Brown adipocytes express only β1 and β3 adrenergic receptors (Bengtsson et al., 2000), although the β-adrenergic receptor profile of subtypes is unknown for the T37i cell line. In contrast, the present study demonstrated that UCP3 mRNA abundance is elevated with *in vitro* non-specific β-adrenergic stimulation of porcine white adipose tissue, suggesting a tissue specific response; as the present study is in agreement with several *in vivo* studies that have reported increases in rodent epididymal UCP3 expression with a variety of β3-adrenergic agonists (Gong et al., 1997; Emilsson et al., 1998; Yoshitomi et al., 1998).

Various *in vitro* experiments have shown that isoproterenol and other β-adrenergic agonists stimulate lipolysis (Mersmann, 1998) and may reduce lipogenesis in porcine adipose tissue (Peterla and Scanes, 1990; Adeola et al., 1992a,b). These metabolic changes with β-adrenergic agonist treatment would indicate that overall fatty acid metabolism is impacted by this treatment. Several studies have suggested that UCP3 may function in the regulation of fatty acid metabolism by shuttling fatty acids across the mitochondrial membrane (Garlid et al., 1998; Harper et al., 2002; Jezek, 2002). Therefore the increase in UCP3 mRNA abundance may be associated with the partitioning of fatty acids with the reported changes in fatty acid metabolism. In conjunction with this, isoproterenol has been demonstrated to promote lipid peroxidation in a variety of tissues (Rathore et al., 1998). A proposed major role for UCP2 in reducing lipoperoxidation within mitochondria has been identified (Jaburek et al., 2004). Thus an increase in UCP2 mRNA abundance with isoproterenol treatment would not be unexpected. Lipoperoxides result from the action of superoxides produced during normal mitochondrial metabolism reacting with a number of molecules to produce reactive oxygen species, such as H_2O_2 (Aikens and Dix, 1993; Droge, 2002; Spiteller, 2002). Previous studies have proposed that UCP3 may function in reducing reactive oxygen species (Negre-Salvayre et al., 1997; Jezek et al., 2004), thus reducing lipoperoxidation. However, further studies are necessary to determine whether isoproterenol and other β-adrenergic agonists alter superoxide production and lipoperoxidation within adipose tissue.

Unlike the *in vitro* experiment with isoproterenol, chronic treatment with ractopamine had no effect on *in vivo* adipose tissue UCP2 or UCP3 mRNA abundance. The limited sensitivity of adipose tissue to ractopamine *in vivo* may be the result of ractopamine's actions to downregulate the β-adrenergic receptor density in subcutaneous adipose tissue relative to skeletal muscle (Spurlock et al., 1994). Thus, the downregulation of β-adrenergic receptors in the adipose tissue may preclude detection of an effect on UCP2 expression *in vivo*. Similarly, adipose tissue UCP3 was unresponsive to ractopamine treatment in the present study, most likely through the same mechanism. The absence of an effect of ractopamine on adipose tissue UCP2 or UCP3 may not be surprising when considering the limited impact of ractopamine on adipose tissue metabolism *in vivo* (Dunshea and King, 1995). Alternatively, the non-specific β-adrenergic agonist isoproterenol may have bound to the limited proportion of β3 adrenergic receptors to elicit the changes in UCP2 and UCP3 mRNA in the *in vitro* experiments, while ractopamine which functions through β1- and β2-adrenergic receptors (Mills and Liu, 1990; Spurlock et al., 1993; Mills et al., 2003) may not elicit a response in UCP2 or UCP3 expression.

Treatment with β2-adrenergic agonists has been demonstrated to increase UCP2 expression in L6 myotubes, a cell line which predominantly expresses β2-adrenergic receptors (Nagase et al., 2001), while treatment of rodents with β3-adrenergic agonists either did not affect skeletal muscle UCP2 expression (Nakamura et al., 2001) or reduced it (Emilsson et al., 1998; Yoshitomi et al., 1998; Berraondo et al., 2000). The dichotomy of response between the experiments with L6 myotubes and skeletal muscle *in vivo* may relate to the differences in the environment of the cells or tissue, the potential role of innervation or differences in β-adrenergic receptor subtype present. The muscle data from the present experiment agrees with the *in vivo* studies.

The apparent reduction in UCP2 expression in both the more oxidative (STR) and glycolytic (STW) portions of the muscle with ractopamine treatment indicates that the causative factors are not related to the metabolic characteristics or fiber composition of the individual portions of the ST, but rather a response of the ST in general. The absence of effect on the LM further suggests muscle specificity in the ST response to ractopamine treatment. Muscle specificity in the UCP response of swine has previously been demonstrated by Damon et al. (2000); T_3 could stimulate UCP3 expression in longissimus but not rhomboideus muscles. However, no differences in serum T_3 or T_4 were detected in the present study. In addition, ractopamine has been demonstrated to produce muscle specific responses in the expression of myosin heavy chain (Depreux et al., 2002); reducing the amount of 2A myosin in STR and STW by more than 20% while not affecting LM. Spurlock et al. (1993) reported no difference in the overall affinity of LM and ST for ractopamine; however this does not exclude possible differences in post receptor events such as activation of adenylate cyclase or desensitization.

Uncoupling protein 3 expression was depressed in STW of ractopamine treated swine in the present study. There was no effect on any other tissue, including the STR. Previous studies in rodents have reported either increases in UCP3 mRNA abundance in rodent skeletal muscle with BRL35135 (Emilsson et al., 1998) or trecadrine (Berraondo et al., 2000), decreases with CL316243 (Yoshitomi et al., 1998; Boss et al., 1999) or BRL37344 (Boss et al., 1999) or no change with short term trecadrine exposure (Gomez-Ambrosi et al., 1999). All of these agents are β3-adrenergic agonists. However BRL37344 may also function through putative β4-adrenergic receptors as mice whose β3-adrenergic receptor gene has been disrupted can still respond to this β-adrenergic agonist. Thus, the variation in response in rodent muscle may depend on the specific treatment selected; the genetics of the animal or the specific muscle selected for analysis.

Another factor which might contribute to a reduction in muscle UCP2 and UCP3 mRNA abundance with ractopamine treatment is the overall changes in physiology/activity of the animal. While the assessed serum hormone and metabolite concentrations were unchanged by ractopamine feeding in the present experiment, ractopamine treatment has been demonstrated to increase the activity level of pigs (Marchant-Forde et al., 2003). Endurance exercise training has been demonstrated to reduce UCP2 and UCP3 mRNA abundance in rat tibialis anterior muscle (Boss et al., 1998). In contrast, acute exercise increased UCP2 and UCP3 mRNA abundance in the rat gastrocnemius muscle (Tsuboyama-Kasaoka et al., 1998). However, the changes in activity in swine with ractopamine treatment cannot be compared to exercise as they comprise primarily increases in standing and feeding activity (Marchant-Forde et al., 2003). Comparison of UCP2 and UCP3 expression in vastus lateralis from able-bodied versus tetraplegic men demonstrated that moderate activity produces lower UCP2 and UCP3 expression relative to inactivity (Hjeltnes et al., 1999). These changes are similar to the present study which may imply a contribution of ractopamine induced changes in activity level with changes in UCP expression; however, the highly specific responses by only selected skeletal muscles in the ractopamine treated pig suggest other factors are contributing to the UCP response.

The detection of differences in UCP3 response between STW and STR in the present study versus the study of Spurlock et al. (2001) may be due to the difference in model (anabolic versus catabolic) or the type of treatment (ractopamine versus fasting). The higher expression of UCP3 in STW than STR would suggest an effect of fiber type on UCP3 expression in the present study, irrespective of the potential role of ractopamine.

Ideally, UCP2 and UCP3 protein would be measured in association with these experiments. However, no UCP3 antibody has been demonstrated to be effective for swine UCP3 (Mostyn et al., 2004) and the only effective UCP2 antibody (Mostyn et al., 2004) has with further propagation been characterized as not cross-reacting well with the pig (Mostyn et al., 2005). Therefore, the relationship between changes in UCP mRNA abundance with β-adrenergic treatment and potential changes in UCP protein concentration cannot be ascertained. Previous studies have demonstrated that UCP2 and UCP3 mRNA and protein levels do not necessarily correlate (Pecqueur et al., 2001; Mostyn et al., 2004). Therefore, caution must be used in interpreting the relationship between changes in porcine UCP mRNA abundance and UCP activity.

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